

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Colostrum and milk can transmit jaagsiekte retrovirus to lambs

This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/100262> since 2016-11-24T13:21:16Z

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

Colostrum and milk can transmit jaagsiekte retrovirus to lambs

Elena Grego^a, Daniele De Meneghi^a, Vega Álvarez^b, Alfredo A. Benito^c,
Esmeralda Minguijón^b, Aurora Ortín^c, Mario Mattoni^a, Bernardino Moreno^b,
Maidier Pérez de Villarreal^c, Alberto Alberti^e, Maria Teresa Capucchio^d,
Marco Caporale^f, Ramón Juste^b, Sergio Rosati^a, Marcelo De las Heras^{c,*}

^a *Dipartimento di Produzioni Animali Epidemiologia ed Ecologia, Facoltà di Medicina Veterinaria,
Università degli Studi di Torino, Grugliasco, Italy*

^b *Instituto Vasco de Investigación y Desarrollo Agrario (NEIKER), Derio, Spain*

^c *Departamento de Patología Animal, Facultad de Veterinaria, Universidad de Zaragoza, Zaragoza, Spain*

^d *Dipartimento di Patologia Animale, Facoltà di Medicina Veterinaria, Università degli Studi di Torino, Grugliasco, Italy*

^e *Istituto di Patologia Speciale e Clinica Medica Veterinaria, Università degli Studi di Sassari, Sassari, Italy*

^f *Institute of Comparative Pathology, Faculty of Veterinary Medicine, University of Glasgow, Glasgow, United Kingdom*

Received 24 October 2007; received in revised form 22 January 2008; accepted 29 January 2008

Abstract

Ovine pulmonary adenocarcinoma (OPA) is a contagious disease caused by jaagsiekte sheep retrovirus (JSRV). In the three studies performed, we have obtained data of the importance of colostrum/milk (C/M) in the transmission of JSRV. In the first study, a group of sheep from a flock with a long history of OPA, samples from colostrum and peripheral blood leucocytes (PBLs) were collected. Two specific PCRs (U3-LTR and *env* of the JSRV) were carried out. Using U3PCR 8/34 sheep were positive in colostrum whereas with *env*PCR 7/34 were positive. From these animals only one was positive with U3PCR in the PBLs. Evidence of the transmission of JSRV infection by C/M was obtained in two more separate studies. In the second study, PBLs from five lambs from JSRV+ ewes and two from JSRV-ewes were tested by the U3PCR. They were fed C/M by their mothers during 3 months and slaughtered 7 months after birth. Three out of five lambs from the JSRV+ sheep became PBL positive at 3–4 months old and the other two were also positive at 4–6 months of age. One lamb of the JSRV-sheep became also PBL positive at an age of 3 months. In the third study, a group of lambs from JSRV negative mothers were fed with C/M from JSRV+ sheep and housed in separate unit. For comparison, another group of the same origin and maintained in another different unit, were fed with C/M containing a JSRV virus preparation. All lambs were blood sampled monthly and JSRV infection was detected as early as 15 days and several times onwards in both groups. Control groups fed with C/M from JSRV free flock and JSRV blood test negative sheep were always negative. Together these results indicate that suckling is an important natural transmission route for JSRV.
© 2008 Elsevier B.V. All rights reserved.

Keywords: Sheep; Jaagsiekte; Retrovirus; Vertical; Transmission; Milk; Colostrum

* Corresponding author. Present address: Institute of Comparative Medicine, Division of Pathological Sciences, Faculty of Veterinary Medicine, University of Glasgow, 464 Bearsden Road, G61 1QH Glasgow, United Kingdom. Tel.: +44 141 330 8478; fax: +44 141 330 5602.

E-mail address: m.delasheras@vet.gla.ac.uk (M. De las Heras).

1. Introduction

Ovine pulmonary adenocarcinoma (OPA, sheep pulmonary adenomatosis, jaagsiekte) is an important infectious disease of sheep that has been reported in many sheep-rearing countries of Europe, Africa, Asia and the America (Sharp and DeMartini, 2003). OPA is a lung tumour derived from type II pneumocytes and Clara cells caused by an exogenous betaretrovirus known as jaagsiekte sheep retrovirus (JSRV) (Palmarini et al., 1999; DeMartini et al., 2001). Most natural cases appear in animals aged 2–4 years and only when the tumour volume becomes sufficient to interfere with lung function, clinical signs can be expected. As the tumour cells are secretory, a considerable amount of fluid can be generated and accumulated in the respiratory tract of the affected animals and the most typical feature of clinical OPA is the discharge of a whitish foamy fluid from the nostrils (De las Heras et al., 2003). The presence of the JSRV can be confirmed in the lung fluid or tumours by immunoblotting (Sharp and Herring, 1983), immunohistochemistry (Sharp et al., 1983; Palmarini et al., 1995; Salvatori et al., 2004), ELISA (Palmarini et al., 1995) and PCR (Bai et al., 1996; Palmarini et al., 1996b). In OPA affected sheep, JSRV is also detectable in cells of the lymphoreticular system, employing a highly sensitive PCR assay (Palmarini et al., 1996b; Holland et al., 1999) or immunohistochemistry (Holland et al., 1999). In spite of this, it is more difficult to identify animals during pre-clinical period due to lack of detectable circulating JSRV-specific antibodies (Sharp and Herring, 1983; Ortín et al., 1998; Summers et al., 2002). However improved PCR techniques have demonstrated JSRV proviral DNA in peripheral blood leucocytes (PBLs) prior to the onset of the neoplasia in experimentally inoculated lambs (Holland et al., 1999). JSRV proviral DNA has been also detected in sheep with no evidence of OPA lesions from commercial flocks with records of clinical cases of OPA (González et al., 2001; De las Heras et al., 2005). The number of PBLs harbouring proviral JSRV DNA is increased in animals with clinical OPA and these animals are more constantly positive (González et al., 2001; De las Heras et al., 2005). Hence, JSRV provirus in cells of the lymphoreticular system can circulate and can be detected irregularly at a very low burden in pre-clinical OPA, while viral load increases when the animal develops the tumour.

OPA can be experimentally reproduced in young lambs in a few weeks, by intra-tracheal inoculation of concentrated lung fluid obtained from naturally affected sheep (Sharp et al., 1983). However, under natural conditions, the disease is believed to have a very long incubation period and the incidence of the clinical disease in affected flocks is generally around 2–5% with most clinical cases in young adult sheep (Sharp and DeMartini, 2003). Epidemiological observations and experience have shown consistently that OPA is transmitted among flocks by movements of apparently unaffected sheep (Sharp and DeMartini, 2003). In fact, the epidemic of OPA in Iceland in 1934 was due to a ram with no visible clinical signs imported from Germany. The respiratory route was considered of great importance in the rapid propagation of that OPA epidemic (Dungal and Gislason, 1938). Thus, natural and experimental evidences support the respiratory route as important in the disease transmission. Moreover, recent studies have indicated that JSRV infection can spread readily both in lambs and adult animals. However, the onset of the clinical disease and tumour lesions during the commercial lifespan of sheep occurs in a minority of JSRV-infected animals after a long incubation period (Caporale et al., 2005). Furthermore, other studies performed under natural conditions indicated that the JSRV infection can be detected in PBLs as early as 2 months in 10–43% of the animals, of which only 8–17% develop OPA (Caporale et al., 2005; De las Heras et al., unpublished observations). The presence of JSRV proviral DNA in blood of very young lambs from PCR positive sheep but with no pathological evidence of OPA, suggests that perinatal transmission route is relevant in the natural transmission of the JSRV infection.

In separate studies, we have shown in this paper that JSRV is detected by PCR techniques in sheep colostrum from a flock with recorded OPA cases and evidences which supports that this virus can be transmitted through maternal route by colostrum and/or milk as a vehicle.

2. Materials and methods

The data presented in this paper have been generated in three different places under the common

objective to find evidences of the presence of JSRV in colostrum, milk and the possible transmission of JSRV from ewe to lambs through the milk and colostrum. In the first study we tested colostrum obtained from ewes from a flock with a long history of OPA by using two different specific PCRs for JSRV. In the second study, we analyzed whether lambs taking colostrum and milk from JSRV blood positive but with no pathological evidence of OPA, became infected by JSRV under natural conditions. Finally, in the third study, we tried to determine whether lambs fed with milk and colostrum from JSRV blood positive mothers or milk/colostrum containing JSRV retrovirus particles from pCMV2JS21 molecular clone produced in the laboratory became infected under separated conditions.

2.1. Detection of JSRV in colostrum from Latxa sheep dairy breed (study 1)

The first data was obtained from a flock of Latxa sheep, a traditional dairy breed from the Basque country in the north of Spain, with an OPA long history. Blood and colostrum samples were taken simultaneously after parturition from 34 sheep belonging to this flock. Peripheral blood leukocytes (PBLs) and somatic cells from colostrum (SC) were obtained and DNA was extracted following previously described procedures (Extramiana et al., 2002) and frozen at -20°C until processed by a modified phenol–chloroform DNA extraction method (Sambrook et al., 1989). One step JSRV U3PCR (U3PCR) (De las Heras et al., 2003) was performed in the blood and colostrum sample from every single animal. In addition, another PCR for the JSRV *env* gene (envPCR) was also carried out only with the colostrum samples. This PCR was developed as a second test for the presence of JSRV in the colostrum samples. The amplicon generated was a 102 bp fragment. The PCR was carried out using 4 pmol of each primer named env A (5' TGGAAAACCTGATTGGTG 3') and env B (CATGGTTTCGCGACTTTCTAAAG 3') in a reaction mix (2.5 mM MgCl_2 , 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 200 μM each dNTP) with 0.75 U of Platinum Taq DNA polymerase (Invitrogen). Each sample was subjected to an amplification profile of 95°C 7 min, 35 cycles of 94°C 30 s, 60°C 30 s and 72°C 30 s, and a final extension of 72°C for 10 min.

Three replicates from each sample, either from blood or from colostrum were tested (300 ng of genomic DNA per 25 μl of reaction mix) and the samples were considered positive when at least one of the replicates was found with evidence of amplified product. DNA extracted from lung tumour lesions of OPA affected animals was used as positive control. Negative controls were included in extraction and amplification of DNA PCR products and analyzed by electrophoresis in a 2% (w/v) agarose gels in Tris–borate–EDTA buffer (TBE), stained with 0.5 mg/ml of ethidium bromide in TBE for 20 min, washed in distilled water for 10 min and visualized on a ultraviolet transilluminator. U3-envPCR negative samples were tested for Glyceraldehyde-3-phosphatase dehydrogenase (GAP-DH) to verify the integrity of the DNA (Palmarini et al., 1996a).

2.2. Detection of JSRV in pre-colostral and post-colostral blood from lambs delivered from JSRV blood positive sheep (Rasa Aragonesa breed) under natural conditions (study 2)

Four pregnant sheep of Rasa Aragonesa breed (meat breed) from a commercial flock in Zaragoza (Spain) with natural cases of OPA and blood PCR positive to JSRV (U3PCR) (De las Heras et al., 2003) (group Z-1) and two more pregnant sheep from the same flock but blood PCR negative (group Z-2) were purchased. The gestation of all sheep was previously synchronised by routine methods and all finally delivered their lambs within 7–10 days interval. To prevent colostrum intake by the lambs, sheep had their udders and nipples covered with a cloth bag from 10 days before expected parturition. This system has been previously used with good results in the control of Maedi-Visna (González and Ruiz, 1995). These sheep delivered nine lambs: five were from JSRV positive sheep and four from JSRV negative sheep, but two lambs from the group Z-2 were stillborn or died shortly after birth. Pre-colostral blood was obtained from lambs as soon as possible and after removing the bras from the udders lambs were allowed to suck. The lambs cohabitated with their mothers for 3 months. By then, all ewes were euthanized following approved procedures by the Committee of Animal Experimentation of the University of Zaragoza, and careful post-mortem examination was carried out. After necropsy,

samples from seven different lung areas for histopathological examination and frozen tissues from mediastinal lymph nodes for PCR studies were taken from all the sheep. The lambs were kept until 7 months of age and during this period all of them were bled four times in the first month of life and every 15 days until the end of the experiment. They were finally slaughtered by the same procedures and taking the same samples as performed in the ewes.

Routine procedures for pathological examination of the tissues were followed and samples from lungs, mediastinal lymph nodes and representative tissues from all organs were fixed in formalin and embedded in paraffin. Representative sections were obtained and stained with standard haematoxylin–eosin techniques. For PCR analysis, samples from frozen tissues were processed and the specific U3PCR for JSRV was carried out as described previously (De las Heras et al., 2003). Three replicates from each sample were tested and they were considered positive when amplification was found at least in one of the replicates. DNA extracted from OPA tumour lesion was used as positive control. DNA extracted from bovine blood was used as negative control. DNA PCR products were analyzed by electrophoresis in a 2% (w/v) agarose gels in TBE, stained with 0.5 mg/ml of ethidium bromide in TBE and visualized on a bioimaging system of automated gel documentation (Gene Genius, Syngene). U3PCR negative samples were tested for GAP-DH to verify the integrity of the DNA (Palmarini et al., 1996a).

2.3. Transmission on artificially feeding lambs from negative JSRV ewe flocks with colostrum/milk from JSRV positive sheep (Biellese breed) (study 3)

2.3.1. Identification, selection and analysis of JSRV/OPA positive sheep (sheep group T-1)

Eighty-three ewes (aged 2–6 years) were randomly selected from two flocks ($N = 900$) with previous OPA clinical records and/or pathological lesions attributable to OPA. They belonged to Biellese breed sheep from flocks located in the Italian northwestern Piedmont. Selected animals were bled twice, DNA was extracted from PBLs and tissues using the Blood DNAeasy Kit (Qiagen) and tested by blood hemi-nested JSRV U3PCR (U3hn PCR) (Palmarini et al., 1996a). Fifteen ewes that were PCR positive in both samplings were re-tested three further times and found

to be persistently infected with JSRV; these were then recruited and included in sheep group T-1. These ewes were used to provide colostrum/milk to feed lambs born from JSRV negative sheep.

Colostrum and milk samples from these ewes were also collected and tested twice (at the beginning and end of the lactation period) in order to detect JSRV in these biological fluids. Fifty milliliters of milk was centrifuged for 10 min at $1000 \times g$ at 4°C . Cream and whey layers removed using a pipette. The somatic cell pellets were re-suspended and washed in 5 ml of phosphate-buffered saline (PBS) centrifuged at $500 \times g$ for 10 min at 4°C . The pellets were then resuspended in 1.5 ml of PBS, transferred to an Eppendorf tube and washed as before. Final pellets were stored at -80°C until DNA extraction. Genomic DNA was extracted from the somatic cell pellets using Blood DNAeasy Kit (Qiagen®) and tested with the U3hn PCR (Palmarini et al., 1996a) and U3PCR (De las Heras et al., 2003).

At the end of the lactation period, JSRV positive ewes were euthanized by using welfare approved protocol, submitted to post-mortem examination and blood, lungs, mediastinal lymph nodes, mesenteric lymph nodes, kidney, liver, spleen and Peyer's patches samples were collected. These tissues were stored at -80°C for subsequent isolation of nucleic acids. The procedures for DNA extraction and PCR analysis used with colostrum samples were also applied to those later samples. Tissues were also processed for histopathological studies following routine procedures.

2.3.2. Identification and selection of JSRV/OPA negative flock (sheep group T-2)

Fifty-three ewes (aged 2–6 years) were randomly selected from a flock ($N = 112$) of the same breed and geographical area but with no previous clinical records and pathological lesions attributable to OPA. Besides, no introduction of foreign breeding stock has been reported in this flock over 8 years period. They were then blood tested twice at two different time points in triplicate by using the JSRV U3hn PCR protocol (Palmarini et al., 1996a) and all were negative.

Ewes selected for this study were kept at the stables/barns of CISRA (Inter-departmental Centre for Animal Care & Housing) Faculty of Veterinary Medicine Grugliasco-Turin, in separated units specifically allocated to them.

2.3.3. Sheep reproductive protocol and lambing management

Ewes from groups T-1 and T-2 were oestrous synchronised and artificial insemination (AI) was performed by laparoscopic technique on all ewes that responded to the synchronization scheme (Manunta et al., 1978). Ewes with progesterone (P4) values higher than 4 ng/ml (as detected by commercial EIA test) were considered pregnant while those with P4 values lower than 4 ng/ml were presented to the ram within the following 24 h. Eighteen lambs from JSRV negative ones were obtained. Immediately after birth, all lambs were taken away from their dams to avoid possible contamination (through licking, suckling, etc.) and were individually identified with rubber collars and coloured/numbered tags. According to the timing of lambing, lambs were allocated to the different experimental lamb groups. Unfortunately some perinatal losses and further deaths which occurred at later stages jeopardised a complete availability of lambs in the four experimental groups throughout the duration of the experimental period (6 months, starting from lambing date).

2.3.4. Lamb groups

Research protocol stipulated that all lambs had to be kept in the experimental premises for at least 6 months after lambing. Lambs included into groups A–D were obtained from JSRV negative ewes (sheep group T-2). Each lamb group was kept in specific boxes allocated to a given group, and physically separated from one other.

Group A: Intra-tracheal inoculation of lambs with a JSRV₂₁ obtained by transient transfection of 293 T cells with a molecular clone of JSRV [pCMV2JS21].

Group A was used as positive control to confirm the infectivity of JSRV₂₁ by intra-cheally route which has been proven to efficiently transmit the infection (Sharp and DeMartini, 2003). Two lambs were infected intra-tracheally with a single dose of JSRV₂₁ obtained from 293 T cell line, transfected with pCMV2JS21 molecular clone. The dose of 2.5 ml corresponded to transfection of 20 Petri dishes (100 mm diameter) of 293 T after viral concentration by ultracentrifugation protocol was carried out essentially as first described by Palmarini et al. (1999). For each dose, 10 µl aliquots of JSRV₂₁ were checked by Western blotting using rabbit serum anti-JSRV major capsid antigen.

Group B: Negative control—three lambs were included in this group, and fed with artificial powdered colostrum (Lamb Volostrum[®], Volac) and milk (Lamlac[®], Volac).

Group C: Oral dosing of lambs with of JSRV₂₁ obtained by transient transfection of 293 T cells with a molecular clone of JSRV [pCMV2JS21]: five lambs were fed for 3 subsequent days with JSRV₂₁ produced as described for group A. A dose of 2.5 ml per animal per day, mixed with equivalent volume of artificial colostrums/milk was administered, the first dose being administered within 6 h after birth.

Group D: Lambs fed with colostrums/milk from JSRV-infected ewes. Eight lambs were bottle-fed with a pool of colostrum/milk milked from JSRV persistently infected ewes (sheep group T-1). As milk from donor ewes was not sufficient to cover diet requirement, artificial powdered colostrums/milk was used as feed supplementation, when required.

Whole-blood samples (EDTA) were drawn from all lambs of the four experimental groups, according to the following sampling protocol—T0: pre-colostrum (within 4–6 h after birth), T1: 15 days after T0; T2: 30 days after T0; T3–T7: after T2, at monthly interval until 6 months of age. Buffy-coat was obtained from whole blood and stored at –80 °C until subsequent DNA extraction, and JSRV U3PCR testing (De las Heras et al., 2005). Each sample was tested in triplicate (1000 ng of genomic DNA per 25 µl reaction mix) and the reactions were considered positive when at least one of the replicas was found to be positive. DNA extracted from sheep with evident lung tumour lesions was used as positive control. Blood and liver of bovine origin were used as negative control and samples were tested at the same time with the test samples. Each control was tested in triplicate. Blood samples from sheep within the groups T1 and T2 were processed equally.

At the end of the study period (6 months from birth), all lambs from the experimental groups were euthanized by using animal welfare approved protocol. Subsequent post-mortem examination and, collection of biological samples from target organs/tissues was carried out. Samples collected to detect the presence of JSRV by U3PCR and for gross and histological investigations were as follows: blood, lungs (apical, medial and caudal pulmonary lobes), liver, kidney, spleen, mediastinal lymph node,

mesenteric lymph nodes and Peyer's patches. Tissues for histological examination were collected immediately after slaughtering, fixed in 10% neutral buffered formalin, and paraffin embedded. Sections (5 µm) were stained with haematoxylin and eosin (H–E). Lambs 11 and 13–15 in the group D died suddenly by other causes before the end of the study and no tissue samples were able to be collected from them (Table 3, Fig. 2).

As a control of integrity of the DNA all samples resulted negative for U3–U3hn PCRs from all samples included in the study 3 were also tested by PCR for GAP-DH (Palmarini et al., 1996a).

Early studies determined the level of sensitivity and specificity of the U3hn and U3PCRs using DNA extracted from sheep blood PBLs. These analyses concluded that when U3hn PCR was performed single but with three replicates the sensitivity was 16.2% and the specificity 98.8%. However, when the U3hn PCR was carried out in the same animal four to five times and with the three replicates each time, the sensitivity reached 50–60% and the specificity was between 91 and 93% (Caporale et al., 2005). Same levels of sensitivity and specificity may be obtained with the U3PCR when testing single sheep because there was a high degree of concordance among both PCRs. In addition, the estimated sensitivity at the flock level with one single U3PCR test in 10–12 animals sampled randomly was 97.5% (De las Heras et al., 2005).

3. Results

3.1. Jaagsiekte retrovirus can be detected in sheep colostrum in flocks with natural cases of OPA or evidence of JSRV infection

Somatic cells obtained from Latxa sheep colostrum samples were positive either in 20.5% using the envPCR or in 23.5% of the samples using the U3PCR. Complementary sensitivity of envPCR relative to U3PCR was 25% (2/8), whereas complementary sensitivity of U3PCR relative to envPCR was 43% (3/7). Both techniques showed moderate agreement according to the Kappa index test (kappa: 0.57; S.E.: 0.1709 (Table 1)). This result indicates that proviral DNA of JSRV can be consistently detected in colostrum from sheep obtained from flock naturally affected by OPA.

Table 1

Results of the JSRV U3PCR and JSRV envPCR in colostrum samples from Latxa breed sheep

envPCR	U3PCR		Total
	Positive	Negative	
Positive	5	2	7
Negative	3	24	27
Total	8	26	34

Kappa test results were—complementary sensitivity: envPCR, 2/8 (25%); U3PCR 3/7 (43%); observed agreement: 0.85; chance expected agreement: 0.65. Kappa: 0.57 (S.E. 0.1709) (study 1).

The JSRV presence in the blood samples from these groups of sheep resulted in a 3% of samples positive using the U3PCR. Furthermore, there were no concordance between the presence of the proviral DNA in somatic cells obtained from colostrum and PBLs obtained simultaneously (Table 2).

3.2. Lambs born from JSRV positive sheep became blood JSRV-PCR positive in a high proportion during early months of life

Pre-colostral blood samples were all negative in both groups of lambs either delivered from JSRV+ (group Z-1) or from JSRV– (group Z-2) mothers. Blood samples taken after providing colostrum in the group Z-1 become positive for the first time in the eighth sample (3 months of age) in one lamb and in the ninth sample (4 months of age) in the other two. Besides, all lambs from this group were positive in one bled at least until the end of the experiment (8 months of age). No OPA lesions were observed in any of the lambs. However, four of them were PCR positive in tissue extracts obtained from mediastinal lymph nodes (Fig. 1).

Table 2

Results of the U3PCR in the blood samples from latxa breed

PCR results in colostrum	PCR results in PBL		Total
	Positive	Negative	
Positive	0	10	10
Negative	1	23	24
Total	1	33	34

Analysis of complementary sensitivity test were: complementary sensitivity: PCR in colostrums, 10/1 (100%); PCR in PBL, 1/10 (10%); observed agreement: 0.676. Chance expected agreement: 0.694. Kappa (S.E.): –0.056 (0.086) (study 1).

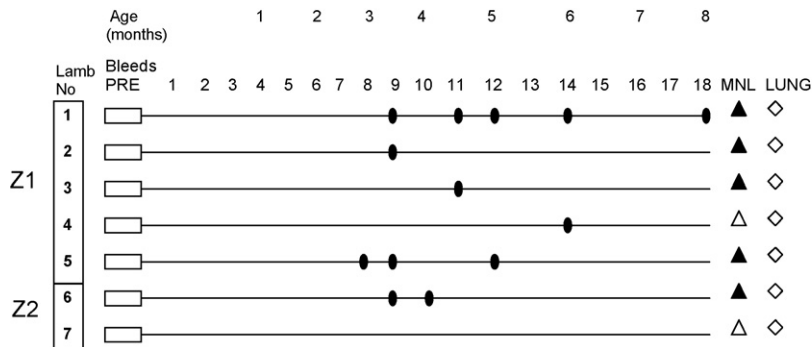


Fig. 1. Detection of JSRV in pre-colostral and post-colostral blood from lambs delivered from JSRV positive and negative mothers under natural conditions (study 2). U3PCR for JSRV in pre-colostral blood samples (rectangles), blood samples from 0 to 8 months of age (ovals), mediastinal lymphnode (triangles) and lung tissues samples (diamonds). Positive results are in black. Lambs 1–5 were delivered from JSRV blood/mediastinal lymphnode positive. Lambs 6 and 7 were delivered from JSRV negative dams.

One lamb of the group Z-2 was positive at 4 month of age (blood sample and also mediastinal lymph nodes) but there were neither OPA lesions nor PCR positivity in the lung samples obtained from it. The other lamb gave PCR negative results in all samples, and no OPA lesions were found in this animal (Fig. 1).

Post-mortem and histopathological examination of the lung samples from all the ewes did not show any evidence of OPA lesions. The presence or absence of JSRV in the blood could be confirmed by the PCR test of the lymphnode tissue extracts of the ewes.

3.3. JSRV proviral DNA can be detected in blood and other organs in lambs artificially fed with colostrum and milk from JSRV-infected sheep

Milk from three OPA positive ewes (group T-1) was tested by JSRV U3hn PCR, and 3/3 ewes were positive, while 2/3 tested by U3PCR were positive in both samples colostrums/milk (at beginning and end of lactation period). Lungs (2/3) and mediastinal lymphnode (1/3) tested by U3PCR were positive. In this group of milk donor sheep, one of them showed gross lesions restricted to small areas of the lungs.

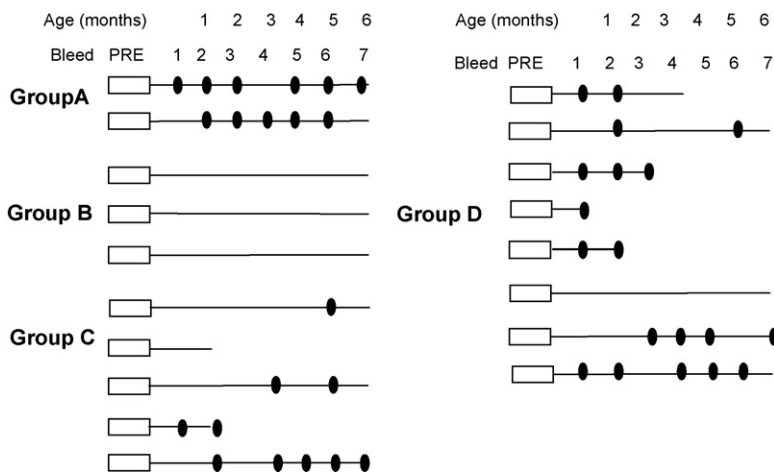


Fig. 2. U3PCR for JSRV in PBLs in the experimental transmission experiments on artificially fed lambs from negative JSRV ewe flocks with colostrum/milk from JSRV positive sheep (study 3)—(group A) intra-tracheal inoculation of a dilution of pCMV2JS21 molecular clone transfected 293 cell line; (group B) negative control; (group C) oral inoculation with a dilution of pCMV2JS21 molecular clone transfected 293 cell line; (group D) lambs fed with colostrums/milk from JSRV infected ewes. Positive results are indicated with black ovals.

Small, white, superficial foci of 1–2 cm in diameter with a solid fibrous, tumour-like appearance were observed in the apical and caudal lobes. Histologically these lesions were compatible with OPA lesions.

After PBLs testing, a total of 2/2 lambs from group A, 4/5 lambs from group C and 7/8 lambs from group D had at least one positive PCR reaction amongst the three replicates tested (Fig. 2). All samples from the negative control (group B) were negative. T0 samples were spoiled by an accident in the laboratory and were not suitable for PCR analysis. More than half, 5/8 of the group D lambs that tested positive for JSRV at least once were positive within the first 1 month after birth. Tissue results were variable: 11/12 lambs exposed to JSRV infection tested positive to at least one tissue. Both lambs inoculated endotracheally were PCR positive to lung tissue (Table 3).

The pathology of group A lambs showed a moderate hypertrophy of the intestinal lymphoid tissue, while neither macroscopic nor microscopic lesions in lungs were observed. Multifocal moderate abnormal proliferation of the alveolar epithelial cells was found, suggesting an initial lesion caused by viral

infection but previously described immunohistochemical tests to detect JSRV matrix and surface proteins were negative (Palmarini et al., 1995).

In lambs groups B and C no significant lesions were observed with the exception of one lamb of group C showing a moderate inflammation of the cranial lobe and an atypical proliferation of the epithelial alveolar cells, limited to restricted areas. In the group D a moderate to severe hyperplasia of mesenteric lymph-nodes and a severe and diffuse hyperplasia of Peyer patches were observed, while no lesions were recorded in the lungs.

4. Discussion

The investigation of the routes of infection of viral diseases in domestic animals is always a critical point in the research of an infectious disease, particularly to implement efficient control measures. OPA clinically affected animals are very easy to diagnose, demonstrating characteristic signs (De las Heras et al., 2003), but JSRV infection in non-clinically affected animal is

Table 3

U3PCR for JSRV in the tissue extracts obtained from lambs in the experiments on artificially fed lambs from negative JSRV ewe flocks with colostrum/milk from JSRV positive sheep (study 3)

Group	Lamb	Lung	Mediastinal Ln.	Mesenteric Ln.	Peyer patches	Spleen	Kidney	Liver
A	1	+	–	+	–	–	–	+
	2	+	+	–	–	+	–	+
B	3	–	–	–	–	–	–	–
	4	–	–	–	–	–	–	–
	5	–	–	–	–	–	–	–
C	6	–	+	–	+	+	–	–
	7	–	+	–	–	–	–	–
	8	–	+	+	–	–	–	–
	9	–	–	–	–	–	–	+
	10	+	–	–	+	+	+	–
D	11	–	–	–	nd	nd	nd	nd
	12	–	–	+	–	–	–	–
	13	nd	nd	nd	nd	nd	nd	nd
	14	nd	nd	nd	nd	nd	nd	nd
	15	nd	nd	nd	nd	nd	nd	nd
	16	–	–	–	–	+	+	–
	17	–	–	–	–	+	–	+
	18	–	–	+	–	–	–	–

Group A: intra-tracheal inoculation of a dilution of pCMV2JS21 molecular clone transfected 293 cell line; group B: negative control; group C: oral inoculation with a dilution of pCMV2JS21 molecular clone transfected 293 cell line; group D: lambs fed with colostrums/milk from JSRV-infected ewes (group T-1). Nd boxes are referred to lambs which died pre-maturely and proper sample collection for analysis was not possible.

very difficult to detect constantly with the existing blood PCR techniques (González et al., 2001; Caporale et al., 2005; De las Heras et al., 2005). These tests are very specific but have a very low sensitivity and because of that they cannot be used for individual testing (Caporale et al., 2005; De las Heras et al., 2005). Furthermore, the number of cells containing the proviral DNA is generally very low (Holland et al., 1999) and its presence is detected irregularly in the blood explaining the very low sensitivity of this test in field studies (González et al., 2001; Caporale et al., 2005; De las Heras et al., 2005). Another constraint to “in vivo” detection of JSRV infection is the lack of either a humoral or a cellular immune response even in clinically affected animals (Ortín et al., 1998; Summers et al., 2002). In spite of these inconveniences, it has been shown that JSRV infection can spread in the progeny of sheep with infection identified by PCR in the blood, but with no clinical signs of OPA (Caporale et al., 2005).

OPA can be transmitted experimentally by inoculating tumour extracts or concentrated lung fluid intratracheally either in sheep or lambs (Sharp and DeMartini, 2003). Furthermore, it is generally accepted that the airborne is the natural transmission route for JSRV because an animal with clinical signs or lung lesions is producing variable amount of fluid containing the virus in high proportions (Sharp and Herring, 1983; Sharp and DeMartini, 2003). However the JSRV dies quickly in the environment (Sharp and DeMartini, 2003), and OPA occurs in a minority of the infected sheep (Caporale et al., 2005), which reduces the possibilities of the spread of the aerogenous infection and do not explain completely why there are many animals infected in the affected flocks (Caporale et al., 2005). Whether infected sheep are disseminating virus or not by breathing even in the absence of tumours is not known.

We have shown in this paper that lambs from blood JSRV positive mothers and with no OPA tumours became infected at very early age. In addition, one of the lambs from JSRV/OPA negative sheep became also JSRV positive in two blood samples and in the mediastinal lymph node after the necropsy. Thereafter, we may consider that the infected sheep were disseminating the virus by breathing and the lambs were infected around birth. However, this event seems to be remote because the virus has been detected in the

lungs in a few infected sheep and was much more detectable in the peripheral blood leucocytes in the same animals (Caporale et al., 2005).

Considering that the virus reservoir in an infected animals seems to be the lymphoreticular system (Palmarini et al., 1996b; González et al., 2001; Caporale et al., 2005) and in agreement with other authors that the lamb infection may occurs perinatally or in the first few months of life (Caporale et al., 2005) maternal routes may be important in the JSRV transmission. Milk transmission is an important route of spread of ovine lentiviruses (Pepin et al., 1998) and for many retroviruses either causing immunodeficiencies (Friendland and Klein, 1987) or tumours (Buffet et al., 1969; Coffin, 1996). Free virus in the plasma or produced in the mammary glands is contained in milk and infects young individuals. In spite JSRV transcripts have been found in several lymphoid organs, it has not been found in the plasma using very specific and sensitive PCR procedures in other studies (Palmarini et al., 1996b). Furthermore, proviral DNA was not found in mammary glands either in OPA clinically affected sheep or in pre-clinical status (González and Ruiz, 1995) and mammary gland tissues obtained from OPA affected animals were negative by immunohistochemistry using a rabbit anti-SU-JSRV (author's unpublished results). We have shown in this paper that colostrum/milk mixed with JSRV viral particles produced in the laboratory can transmit the infection (group C in the study 3), however the possibilities of transmission of JSRV infection by colostrum/milk containing free virus under natural conditions have to be investigated.

Another way for JSRV transmission to lambs using as a vehicle the sheep colostrum or milk may be represented by lymphoid cells or macrophages containing the virus. We have mentioned that proviral DNA JSRV and RNA transcripts can be found in T, B lymphocytes and macrophages obtained from PBL and several lymphoid organs (Palmarini et al., 1996a). It is well known that colostrum is very rich in these cells because they are very important for the lamb protection against external pathogens. These cells from the mother transferred with the colostrums can pass the lamb intestinal barrier and colonize mesenteric lymphoid tissues in the lamb (Tuboly and Bernáth, 2002). These cells may act as a “Trojan horse” to introduce the JSRV into the lamb organism.

We have presented PCR evidence of JSRV in somatic cells obtained from colostrum in a sheep flock with long history of OPA. Furthermore, we have seen that feeding lambs from negative flocks with colostrum and milk obtained from JSRV positive ewes can transmit the infection. These animals showed signs of permanent infection long enough after birth to exclude mother cells circulating in their lambs. In addition, the possibility of an airborne infection from the mothers seemed very low because all lambs were separated from their mothers at birth. Thus, we may conclude that colostrum and milk can transmit JSRV. If this event is important in the transmission of the JSRV, the infection may be stopped by heat treatment of colostrum, because after this treatment lymphoid cells are not absorbed in lamb intestines (Tuboly and Bernáth, 2002). In fact, in a recent study conducted in Germany in a flock with high level of OPA, the same control measures to control Maedi-Visna were implemented, creating a new flock with lambs separated at birth from the mothers and artificially feeding colostrum and milk. They could not eliminate completely the infection but the disease was dramatically reduced (Voigth et al., 2007).

The possibility of the germ line infection or transplacental transmission of JSRV must not be excluded but the importance of this route remains controversial. Sheep placenta is synepithelialchorial where the separation of the maternal–fetal components is pronounced and possibilities of JSRV transmission seems to be difficult. In spite of this, in a study, some of the foetuses obtained from animals which showed clinical signs of OPA were positive (De las Heras et al., 2001). However, in other studies, foetuses obtained from 20 JSRV positive sheep, but with no signs of OPA, were all negative (Caporale et al., 2005).

More studies must be developed to determine how JSRV is transmitted to lambs via colostrum and milk, and how it can develop a durable infection. However, in this paper we have presented data indicating that this route of infection may be important. Taking into account these evidences, the provision of heated colostrums and milk substitutes to lambs as those in use to control Maedi-Visna infection can be proposed initially as a control measures of JSRV and OPA.

Acknowledgements

Authors would like to thank: personnel of CISRA for collaboration and general logistic/technical support; the Livestock Extension services, Agriculture Office, Province of Siena, for design/management of the reproduction protocol, and the Div. Animal Reproduction & Obstetrics; Dept. Animal Pathology, for assistance during lambing; the colleagues & laboratory technicians, Div. Infectious Diseases; Massimo Palmarini, Institute of Comparative Medicine, University of Glasgow, for providing the molecular clone and farmers and veterinary surgeons from Italy and Spain for their very appreciated help; staff of the “Servicio de Apoyo a la Experimentación Animal” of the Universidad de Zaragoza, Spain for their help. This work has been financed by the UE project QLK2-CT-2001-02380.

References

- Bai, J., Zhu, R.Y., Stedman, K., Cousens, C., Carlson, J., Sharp, J.M., DeMartini, J.C., 1996. Unique long terminal repeat U3 sequences distinguish exogenous jaagsiekte sheep retroviruses associated with ovine pulmonary carcinoma from endogenous loci in the sheep genome. *J. Virol.* 70, 3159–3168.
- Buffet, R., Grace, J., Mirand, E., 1969. Vertical transmission of murine leukemia virus. *Cancer. Res.* 29, 588–595.
- Caporale, M., Centorame, P., Giovannini, A., Sacchini, F., Di Ventura, M., De las Heras, M., Palmarini, M., 2005. Infection of lung epithelial cells and induction of pulmonary adenocarcinoma is not the most common outcome of naturally occurring JSRV infection during the commercial lifespan of sheep. *Virology* 338, 144–153.
- Coffin, J.M., 1996. *Retroviridae, The Viruses and their Replication*, 3rd ed. Raven Press, New York.
- De las Heras, M., Garcia-Goti, M., Ortin, A., Pascual, Z., Minguijón, E., Ferrer, L.M., Garcia de Jalón, J.A., Dewar, P., Sharp, J.M., Gonzalez, L., 2001. Presencia del retrovirus asociado a la adenomatosis pulmonar ovina en fetos y en una neoplasia pulmonar en un cordero de tres días. *Pequeños Ruminantes* 1, 36–37.
- De las Heras, M., Gonzalez, L., Sharp, J.M., 2003. Pathology of ovine pulmonary adenocarcinoma. *Curr. Top. Microbiol.* 275, 25–54.
- De las Heras, M., Ortín, A., Salvatori, D., Pérez de Villareal, M., Cousens, C., Ferrer, L.M., Cebrián, L.M., García de Jalón, J.A., González, L., Sharp, J.M., 2005. A PCR technique for the detection of Jaagsiekte sheep retrovirus in the blood suitable for the screening of ovine pulmonary adenocarcinoma in field conditions. *Res. Vet. Sci.* 79, 259–264.

- DeMartini, J.C., Bishop, J.V., Allen, T.E., Jassim, F.A., Sharp, J.M., De Las Heras, M., Voelker, D.R., Carlson, J.O., 2001. Jaagsiekte sheep retrovirus proviral clone JSRV (Js7), derived from the Js7 lung tumour cell line induces ovine pulmonary carcinoma and it is integrated into the surfactant protein A gene. *J. Virol.* 75, 4239–4246.
- Dungal, N., Gislason, G., 1938. Epizootic Adenomatosis in the lungs of sheep—comparison with Jaagsiekte, verminous pneumonia and Progressive pneumonia. *J. Comp. Path. Therap.* 51, 46–48.
- Extramiana, A.B., González, L., Cortabarria, N., García-Goti, M., Juste, R., 2002. Evaluation of a PCR technique for the detection of Maedi-Visna proviral DNA in blood, milk and tissue samples of naturally infected sheep. *Small Rumin. Res.* 44, 109–117.
- Friendland, G.H., Klein, R.S., 1987. Transmission of the human immunodeficiency virus. *N. Engl. J. Med.* 317, 1125–1135.
- González, L., Ruiz, A., 1995. Maedi-Visna una importante enfermedad ovina y su control. Monografías de divulgación, Servicio de Investigación y Mejora Agraria y Consejo Superior de Investigaciones Científicas, Bilbao, Spain.
- González, L., García-Goti, M., Cousens, C., Dewar, P., Cortabarria, N., Extramiana, A.B., Ortín, A., De Las Heras, M., Sharp, J.M., 2001. Jaagsiekte sheep retrovirus can be detected in the peripheral blood during the pre-clinical period of sheep pulmonary adenomatosis. *J. Gen. Virol.* 82, 1355–1358.
- Holland, M.J., Palmarini, M., García-Goti, M., González, L., McKendrick, I., De las Heras, M., Sharp, J.M., 1999. Jaagsiekte retrovirus is widely distributed both in T and B lymphocytes and in mononuclear phagocytes of sheep with naturally and experimentally acquired pulmonary adenomatosis. *J. Virol.* 73, 4004–4008.
- Manunta, G., Nuvole, P., Cappai, P., 1978. Ulteriori indagini sull'induzione ormonale del parto negli ovini. *Arch. Vet. Ital.* 29, 41–45.
- Ortín, A., Minguijón, E., Dewar, P., García, M., Ferrer, L.M., Palmarini, M., González, L., Sharp, J.M., De las Heras, M., 1998. Lack of a specific immune response against a recombinant capsid protein of Jaagsiekte sheep retrovirus in sheep and goats naturally affected by enzootic nasal tumour or sheep pulmonary adenomatosis. *Vet. Immunol. Immunopathol.* 61, 229–237.
- Palmarini, M., Dewar, P., De las Heras, M., Inglis, N.F., Dalziel, R.G., Sharp, J.M., 1995. Epithelial tumour cells in the lungs of sheep with pulmonary adenomatosis are major sites of replication for Jaagsiekte retrovirus. *J. Gen. Virol.* 76, 2731–2737.
- Palmarini, M., Cousens, C., Dalziel, R.G., Bai, J., Stedman, K., DeMartini, J.C., Sharp, J.M., 1996a. The exogenous form of Jaagsiekte retrovirus is specifically associated with a contagious lung cancer of sheep. *J. Virol.* 70, 1618–1623.
- Palmarini, M., Holland, M.J., Cousens, C., Dalziel, R.G., Sharp, J.M., 1996b. Jaagsiekte retrovirus establishes a disseminated infection of the lymphoid tissues of sheep affected by pulmonary adenomatosis. *J. Gen. Virol.* 77, 2991–2998.
- Palmarini, M., Sharp, J.M., De las Heras, M., Fan, H., 1999. Jaagsiekte sheep retrovirus is necessary and sufficient to induce a contagious lung cancer in sheep. *J. Virol.* 73, 6964–6972.
- Pepin, M., Vitu, C., Russo, P., Mornex, J.F., Peterhans, E., 1998. Maedi-Visna virus infection in sheep: a review. *Vet. Res.* 29, 341–367.
- Salvatori, D., González, L., Dewar, P., Cousens, C., De las Heras, M., Dalziel, R.G., Sharp, J.M., 2004. Successful induction of ovine pulmonary adenocarcinoma in lambs of different ages and detection of viraemia during the preclinical period. *J. Gen. Virol.* 85, 3319–3324.
- Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor, New York.
- Sharp, J.M., DeMartini, J.C., 2003. Natural history of JSRV in sheep. *Curr. Top. Microbiol.* 275, 55–80.
- Sharp, J.M., Herring, A.J., 1983. Sheep pulmonary adenomatosis: demonstration of a protein which crossreacts with the major core proteins of Mason–Pfizer monkey virus and Mouse mammary tumour virus. *J. Gen. Virol.* 64, 2323–2328.
- Sharp, J.M., Angus, K.W., Gray, E.W., Scott, F.M., 1983. Rapid transmission of sheep pulmonary adenomatosis (jaagsiekte) in young lambs. *Arch. Virol.* 78, 89–95.
- Summers, C., Neill, W., Dewar, P., González, L., van der Molen, R., Norval, M., Sharp, J.M., 2002. Systemic immune responses following infection with Jaagsiekte sheep retrovirus and in the terminal stages of ovine pulmonary adenocarcinoma. *J. Gen. Virol.* 83, 1753–1757.
- Tuboly, S., Bernáth, S., 2002. Intestinal absorption of colostral lymphoid cells in newborn animals. *Adv. Exp. Med. Biol.* 503, 107–114.
- Voigth, K., Krämer, U., Brüggmann, M., Dewar, P., Sharp, J.M., Ganter, M., 2007. Eradication of ovine pulmonary carcinoma by motherless rearing of lambs. *Vet. Rec.* 161, 129–132.